



Review

A review on microbial lipids as a potential biofuel

Sara A. Shields-Menard^a, Marta Amirsadeghi^b, W. Todd French^c, Raj Boopathy^{a,*}

^a Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA

^b Department of Chemical and Materials Engineering, California State Polytechnic University, Pomona, CA 91768, USA

^c Dave C. Swalm School of Chemical Engineering, Mississippi State University, Mississippi State 39762, USA

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ABSTRACT

Energy security, environmental concerns, and unstable oil prices have been the driving trifecta of demand for alternative fuels in the United States. The United States' dependence on energy resources, often from unstable oil-producing countries has created political insecurities and concerns. As we try to gain energy security, unconventional oil becomes more common, flooding the market, and causing the major downshift of the usual unstable oil prices. Meanwhile, consumption of fossil fuels and the consequent CO₂ emissions have driven disruptions in the Earth's atmosphere and are recognized to be responsible for global climate change. While the significance of each of these three factors may fluctuate with global politics or new technologies, transportation energy will remain the prominent focus of multi-disciplined research. Bioenergy future depends on the price of oil. Current energy policy of the United States heavily favors petroleum industry. In this review, the current trend in microbial lipids as a potential biofuel is discussed.

1. Introduction

Petroleum is one of the major energy sources consumed in the United States. Seventy-one percent of petroleum supply is fed directly to the transportation sector, supplying over 90% of transportation's liquid fuel (Fig. 1). Some scientists have predicted that we will run out of petroleum in the next 50 years, as we are consuming fossil fuels at an alarming rate (Soetaert and Vandamme, 2009). While this doomsday argument may increase the urgency for alternatives to conventional petroleum reserves, unconventional drilling, such as hydraulic fracturing and horizontal drilling, have made harder-to-get oil possible; thereby increasing the US's oil production tremendously. Hydraulic fracturing, or fracking, involves pumping water, sand, and potentially hazardous chemicals into gas shale (deep underground layers of rock) to release oil and natural gas (Conti et al., 2014a). Recent advances in horizontal drilling have enabled drillers to extract even more gas and oil from miles of shale instead of just a small section. This newfound abundance of oil has impacted global oil prices and triggered uncertainty in the future of biofuel research and commercialization.

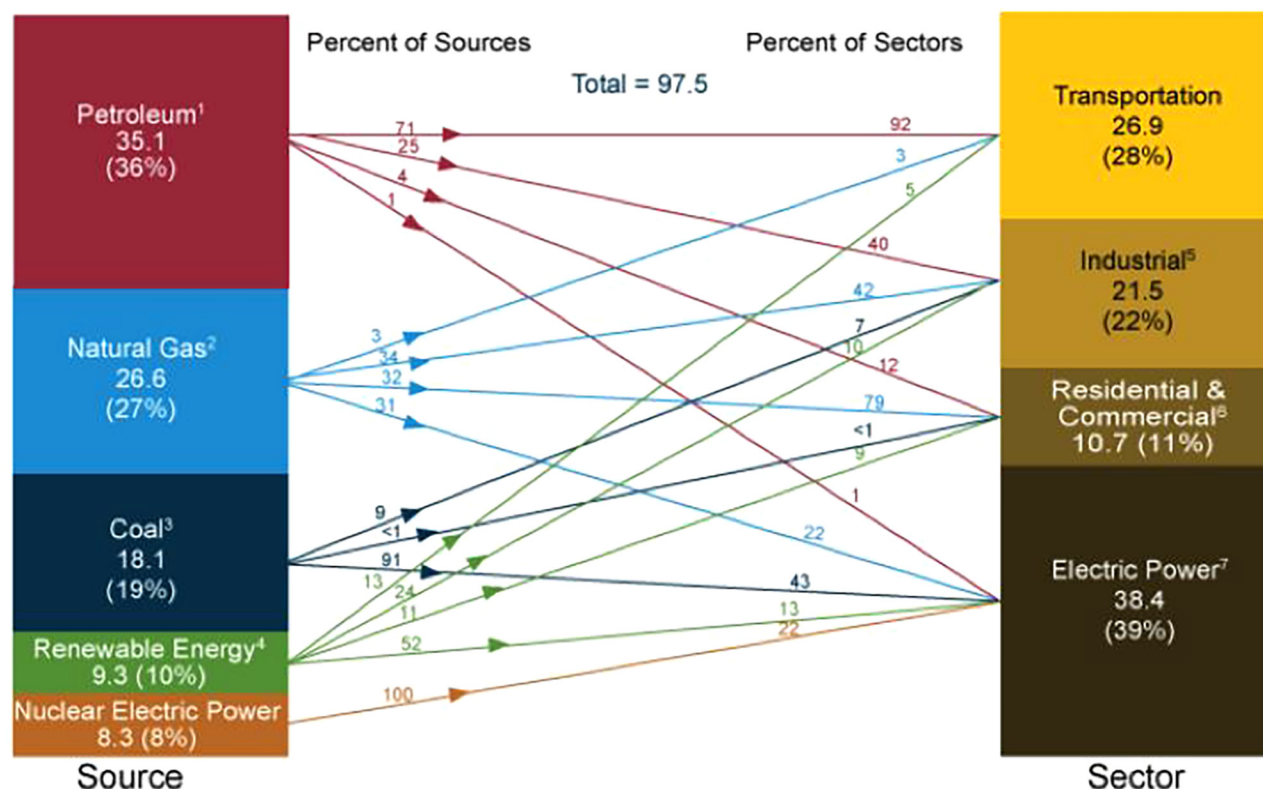
In 2018 the US is poised to be one of the largest oil producers in the world with a predicted 2018 output of 10.3 million barrels per day, surpassing the US record set in 1972 (EIA, 2018). This increase in production coincides with the collapse of oil prices, from \$107/barrel to below \$50/barrel that occurred in the fall of 2014 and continued into 2015 (Bloomberg, 2015). Supply and demand as well as global

instabilities drives the price of oil. As oil production increased, demand declined, particularly in the US, where more fuel-efficient vehicles and better driving practices (less miles, public transportation, etc.) caused an 8% decrease in gasoline consumption (Bloomberg, 2015). In addition to the oil usage in the US, largely driven by the increase in unconventional drilling, the Organization for Petroleum Exporting Countries (OPEC), which controls 40% of the market, has not limited their production, causing a buildup of inventory with a decline in demand (EIA, 2015b). Historically, Saudi Arabia, OPEC's largest producer, would cut some of its oil production to stabilize the market. Now, however analysts indicate that OPEC is threatened by the US's shale oil dominating the market and would rather take the price cuts than relinquish market control to the US or other Persian Gulf producers (Lane, 2015; McGee, 2014; Mirhaydari, 2015; Solomon and Said, 2014; Waldman, 2015).

Oil production and thereby oil prices, are also affected by geopolitical instabilities and also change in government policies due to change in government. In 2008, energy security was one of several driving factors of the development of alternative fuels that would allow the US to be self-sufficient in energy production (Lopes, 2015). Energy security can also pertain to mitigating disruptions in oil supplies. In January 2015, non-OPEC supply was disrupted by over a half million barrels per day due to conflicts in South Sudan, Syria, and Yemen (EIA, 2015a). Furthermore, OPEC production in Libya, Angola, Algeria, and Kuwait declined and production in Iraq and Iran is expected to be disrupted by

* Corresponding author.

E-mail address: Ramaraj.boopathy@nicholls.edu (R. Boopathy).



Endnotes:

¹Does not include biofuels that have been blended with petroleum---biofuels are included in "Renewable Energy."

²Excludes supplemental gaseous fuels.

³Includes less than -0.1 quadrillion Btu of coal coke net imports.

⁴Conventional hydroelectric power, geothermal, solar/PV, wind, and biomass.

⁵Includes industrial combined-heat-and-power (CHP) and industrial electricity-only plants.

⁶Includes commercial combined-heat-and-power (CHP) and commercial electricity-only plants.

⁷Electricity-only and combined-heat-and-power (CHP) plants whose primary business is to sell electricity or electricity and heat, to the public. Includes 0.2 quadrillion Btu of electricity net imports not shown under "Source."

Note: Primary energy in the form that it is first accounted for in a statistical energy balance, before any transformation to secondary or tertiary forms of energy (for example, coal is used to generate electricity).

*Sum of components may not equal total due to independent rounding.

Fig. 1. Primary energy consumption by source and sector in 2013. Units are in quadrillion Btu. Source EIA, 2014.

the increasing activity and threat of the Islamic State of Iraq and the Levant (EIA, 2015a). According to the Truman National Security Project, clean energy development is critical to reducing our dependence on a volatile global oil market and mitigating the national security threat of climate change (TSBB, 2014). Thus, the global energy economy is controlled by various factors leading to volatility in the market. This review provides a current status of biofuel research with special emphasis on microbial lipids as a potential bioenergy source.

2. Environmental concerns

Drilling practices and petroleum-related emissions have led to serious environmental concerns. Shale drilling can access natural gas and oil with a lower carbon footprint (Lopes, 2015) and in displacing/replacing coal plants, shale has also contributed to reducing CO₂ emissions in the US (Carey, 2012). More recently scientists with the US

Geological Survey (USGS) have observed "induced earthquakes" from the fluid-injection methods of shale drilling in areas of the US that have historically been geologically stable (Ellsworth, 2013; McGarr et al., 2015). For example, in 2014, the rate of 3 magnitude or greater earthquakes was greater in Oklahoma than in California (McGarr et al., 2015; McNamara et al., 2015). Induced earthquakes in the US may be a result of the deep disposal of wastewater or fluids related to oil and gas. New regulations for wastewater disposal, better fault monitoring systems and data sharing will help to improve our understanding of the role of fracking and induced earthquakes (McNamara et al., 2015).

Another environmental concern associated with unconventional drilling is groundwater contamination in many states in the US that have fracking activities. A study in 2013 found that methane concentrations in shallow residential drinking-water wells were 6 times higher than in homes farther away from the drill site (Jackson et al., 2013). Some European countries have chosen not to use shale gas until

these environmental concerns are addressed (Patel and Viscusi, 2013). The US Environmental Protection Agency (EPA) released a draft of a three-year study on the impact of fracking on drinking water and found that while there is potential for fracking activities to impact drinking water resources, there was no evidence that fracking had a widespread impact as the number of instances were small compared to the amount of active fracking wells (EPA, 2015). The EPA is still investigating safe disposal methods for the large volumes of wastewater produced with shale gas extraction.

Fossil fuel emissions and wild fires have contributed to the increase in atmospheric CO₂ concentrations (Peters et al., 2007). Oceans act as a CO₂ sink, absorbing almost 26% of CO₂ emissions (SCOPE, 2015). As CO₂ concentrations rise and oceans are increasingly absorbing CO₂, water surface temperature increases and ocean acidification occurs, thereby potentially reducing the capacity of the Earth to mitigate rising CO₂ emissions (Le Quere et al., 2010; SCOPE, 2015). A recent study by Feldman et al. (2015) concluded that the greenhouse effect was indeed due to anthropogenic emissions. Observed evidence showed that rising CO₂ concentrations affect the Earth's surface energy and are a direct result of anthropogenic climate change and variations in photosynthetic activity (Feldman et al., 2015). The International Energy Agency issued its World Energy Outlook report predicting that long-term global temperatures will increase by 3.6 °C (IEA, 2014). To prevent the widespread effects of global climate change, the International Panel on Climate Change recommends limiting temperature increase to 2 °C, which means that the world cannot emit over 1,000 gigatons of CO₂ after 2014 (Conti et al., 2014b; IEA, 2014). As emissions are not likely to be reduced to zero, even with these recommendations, further alternatives such as cleaner burning fuels are necessary to mitigate the onset of global climate change.

3. The current status of biofuels

3.1. The renewable fuel standard

The Energy Policy Act of 2005 authorized the first Renewable Fuel Standard (RFS), which would mandate a minimum volume of biofuels for national transportation use (EPA, 2005). In 2007 the Energy Independence and Security Act (EISA) was established to further reduce energy dependence and increase national security, to stimulate the economy by creating jobs, and to initiate research for producing clean, renewable, and efficient fuels (EISA, 2007). The EISA revised RFS1 and established RFS2, which mandated that 36 billion gallons of renewable fuel be produced by 2022, including 15 billion gallons of corn-based ethanol, 16 billion gallons of cellulosic biofuels, and 1 billion gallons of biodiesel (EISA, 2007). The US Environmental Protection Agency regulates the RFS through renewable identification numbers, compliance standards, and waivers for industries producing total renewable fuels, including advanced biofuels, cellulosic biofuels, and biomass-based diesel (Schnepf and Yacobucci, 2013). However, there has been significant instability of the RFS policy, including a delay by the EPA in setting the 2014 renewable fuel volume obligations. The Biotechnology Industry Organization (BIO) cites that this policy instability has scared off capital investments and cost 80,000 jobs that would have been created by the industry (BIO, 2014). Furthermore, an estimated 21 million metric tons of CO₂ equivalent, equal to 4.4 million additional cars on the road, is the difference between a consistent RFS and policy instability (BIO, 2014). The American Petroleum Institute (API) argues that the RFS is a broken policy, largely citing the blend wall (market's ability to absorb the amount of biofuels produced) and its effect on supply disruptions and cost increases (API, 2015). A study by BIO concludes that in the 10-year span of the RFS, US carbon emissions have been reduced by 589.33 million metric tons (equal to over 124 million cars on the road) and almost 1.9 billion barrels of foreign oil have been displaced (BIO, 2014). The RFS began as a catalyst of the US's advancement of renewable fuels and is now a highly debated policy with

significant implications for the future of the biofuels industry. Current policies of the US government heavily favor petroleum industries with relaxed environmental regulations for drilling. With the oil price hovering around \$ 60 a barrel, the investment in biofuel industry was set back with less investment in research and development and commercialization.

3.2. The generations of biofuels

In response to the increasing need for biofuels and the establishment of the RFS, new technologies, processes, and renewable biological sources are being rapidly developed. Generations are used to chart the evolution of the biofuels industry as research and commercialization progress. Conventional biofuels, also known as first generation, include processes that are already producing biofuels on a commercial scale, such as sugar and starch based ethanol and plant oil biodiesel (IEA, 2015). The benefits of first generation biofuels include economic and social security as well as environmental benefits (Naik et al., 2010). A popular example of first generation biofuels is bioethanol. Bioethanol is generally produced from the fermentation of sucrose from either sugarcane or sugar beets or from the fermentation of sugars derived from the enzymatic digestion of corn starch (Granda et al., 2007). While the use of ethanol for transportation fuel is not new, the growth of ethanol production has significantly increased, tripling yields between 2000 and 2007 (Granda et al., 2007).

However, to be a viable alternative to fossil fuels, Hill et al. (2006) proposed that the fuel should have better environmental benefits, provide a net energy gain, and be economically competitive without government subsidies. After years of research on the implications of first generation biofuels, competition for land and water used for food production (food vs. fuel) is often listed as the primary disadvantage (Graham-Rowe, 2011; Naik et al., 2010; Sims et al., 2010). The worldwide demand for both food and transportation fuel challenge the significance of food-based biofuels (Hill et al., 2006; Tao and Aden, 2009). For example, in 2007 the US planted over 90 million acres of corn, but 30% was used for ethanol production and not for food (Graham-Rowe, 2011). This land use displacement increased food prices and spurred land use competition in an attempt to satisfy national energy demands (Hill et al., 2006; Tao and Aden, 2009). In 2015, as food production is estimated to require a 60% increase by 2050, the Food and Agricultural Organization (FAO) has shifted its focus from a "food vs fuel" debate to a "food and fuel debate", citing both as priorities for the future (UN, 2015).

Advanced biofuels include the later generations, which are still in the research and development or pilot phase. Second generation biofuels, or advanced biofuels, are produced from non-food biomass in an effort to mitigate the food vs. fuel debate created by first generation biofuels. These biofuels are produced in a more sustainable way, which some claim is truly carbon neutral or carbon negative toward its impact on CO₂ emissions (Naik et al., 2010). Lignocellulosic biomass is the feedstock for second generation biofuels and consists of crop by-products or energy crops, such as switchgrass and energy cane. Energy crops require less fertilizer inputs and grow on marginal lands, resulting in a greater environmental benefit (Hill et al., 2006). Furthermore, although competing with food for land use, lignocellulosic biomass energy yields (GJ/ha) are expected to be greater than if first generation crops were grown on the same land (Sims et al., 2010). In 2007 the US, along with the private sector, allocated \$385 million to the US Department of Energy Biomass Program to research and develop several large-scale ethanol demonstration plants (Sims et al., 2010). Despite the large investments, several technical challenges remain towards the cost-effective and efficient utilization of lignocellulosic biomass.

Other advanced biofuel generations include third generation biofuels that produce a feedstock as well as a fuel, such as algae, and fourth generation biofuels that consist of electrofuels (Lane, 2010). These generations are still in the very early stages of technology development

and have high capital costs. Second generation technologies are just now emerging as commercially viable, as POET-DSM Advanced Biofuels opened the first corn stover cellulosic ethanol plant in the US in September 2014 (Thorp et al., 2015). The successful development and implementation of advanced biofuels using non-food biomass could provide 27% of the total US transport fuel by 2050 while decreasing CO₂ emissions by 20% or 2.1 Gt (IEA, 2011).

3.3. The lignocellulosic challenge

Lignocellulosic biomass is the most abundant and renewable organic material in the world (Claassen et al., 1999). Examples of lignocellulosic biomass include wood, grass, forestry waste and agricultural residues (Palmqvist and Hahn-Hagerdal, 2000). The advantages of lignocellulosic residues over food crops are that lignocellulosic material does not compete with food economics and does not require extensive environmental resources. The lignocellulosic complex is composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose account for 70% of dry cellulosic material, which makes biomass ideal feedstocks for biofuels as the sugars derived from cellulosic material can be used as substrate for microbial fermentation (Claassen et al., 1999; Franden et al., 2009; Palmqvist and Hahn-Hagerdal, 2000; Pandey and Kim, 2011).

Several pretreatment methods have been developed to free the cellulose, which consists of glucose units, and hemicellulose, which consists of pentose and hexose sugars, mainly xylose (Hu and Ragauskas, 2012; Mosier et al., 2005). As a result of lignocellulosic pretreatment, inhibitors such as acids, furans, and phenolic compounds are present in lignocellulosic hydrolysate and can affect fermentation productivity (Palmqvist and Hahn-Hagerdal, 2000). While cellulose and hemicellulose can be used to produce various bioproducts, lignin is a complex polyaromatic structure that is often recalcitrant to most bacteria and therefore limited in its use as a renewable and valuable resource.

Furfural and acetic acid have been shown to significantly impact the yield of microbial fermentations, which is more widely documented for ethanol-producing microbes. As concentrations of acetic acid, formic acid, and levulinic acid increased, yeast ethanol yield decreased (Larsson et al., 1998). Acetate and furfural decreased the growth rates of *Zymomonas mobilis*, an ethanol-producing bacterium (Franden et al., 2009). Even if inhibitors do not affect ethanol yields, the growth rates and ethanol production rates can be inhibited by furans and aromatic compounds present in hydrolysates (Klinke et al., 2004). Thus, the challenge of using lignocellulosic biomass for microbial conversion to a product lies in the ability of an organism to utilize pentose and hexose sugars while surviving in the presence of chemical inhibitors.

Lignin accounts for 10–25% of lignocellulosic material and the global paper industry alone produces almost 50 million tons of lignin per year (Gosselink et al., 2004). Only 2% of lignin is used commercially while the remainder is usually burned for energy; however, conversion of lignin offers potential for the microbial production of alternative fuels or bioproducts (Kosa and Ragauskas, 2012; Zakzeski et al., 2010). Lignocellulosic pretreatment while freeing sugars for fermentation, also degrades lignin to lignin oligomers and phenolic derivatives, such as vanillic acid, trans-p-coumaric acid and 4-hydroxybenzoic acid (Palmqvist and Hahn-Hagerdal, 2000; Saadia and Ashfaq, 2010), which sometimes inhibit fermentation process depending on the biocatalyst used in the system. Degraded lignin fractions are often discarded due to lack of use and are ultimately found in industrial wastewaters (Saadia and Ashfaq, 2010; Upadhyaya et al., 2013; Wells et al., 2015). Alternatively, as cellulosic biomass refineries will produce more lignin than can be used for power (by burning), researchers are interested in transforming the excess lignin to value-added products, such as carbon fiber, plastics, and other chemicals currently being made from petroleum (Ragauskas et al., 2014).

3.4. Biodiesel

Biodiesel is a diesel fuel alternative made from renewable sources of triacylglycerides (TAGs) commonly found in vegetable oils and animal fats (Ma and Hanna, 1999; Tao and Aden, 2009). Transesterification is the common method in the US to make biodiesel by reacting a fat or oil with an alcohol to form fatty acid methyl or ethyl esters (FAMES/FAEEs) and glycerol (Bart et al., 2010; Ma and Hanna, 1999). Ethanol or methanol are the most frequently used alcohols for transesterification due to their low cost and quick reactions and a catalyst can also be used to improve the rate and yield of the reaction (Ma and Hanna, 1999). Different feedstock oils can affect the properties of the final biodiesel product, such as the cetane number, oxidative stability, and viscosity, which can ultimately have positive or negative impacts on biodiesel performance (Ciolkosz, 2009).

Soybean biodiesel, one of the most predominant alternative fuels in the US, reduces several air pollutants as well as greenhouse gases by 41% compared to diesel (Hill et al., 2006). Biodiesel also produces 93% more usable energy than its required input of fossil fuels compared to corn ethanol, which only has a 25% net gain of usable energy (Hill et al., 2006). Despite the growing interest in biodiesel, it is not economically viable as almost 85% of its cost lies in feedstock costs (Canakci and Sanli, 2008; Pinzi et al., 2013). Furthermore, biodiesel from edible oils, such as vegetable oils (soybean, sunflower, palm, etc.), is blamed for the increase in food prices and the abundant agricultural inputs for crop cultivation (Leiva-Candia et al., 2014). However, other researchers argue that soybeans are nitrogen fixers, requiring less agricultural inputs and are grown primarily for its protein with oil as a by-product (Granda et al., 2007). Soybean biodiesel is a promising fuel alternative, but feedstock costs and availability (Tao and Aden, 2009), fertile land requirements, and agricultural input are cost-limiting factors in its production (Hill et al., 2006). Therefore, alternative feedstocks and fuel sources must be studied.

Non-edible feedstock alternatives for biodiesel include waste frying oils, animal fats, algae oil including microalgae, and oils from microorganisms. Waste frying oil and animal fats have had some success as a feedstock for biodiesel, but the high content of free fatty acids results in biodiesel with high viscosity (Pinzi et al., 2013). Algae, yeasts, and bacteria can accumulate oil during optimal cultivation conditions and have several advantages over plant oils. These organisms have faster growth rates and are generally not affected by seasonal changes (Li et al., 2008). Challenges to microbial oil production remain in understanding the fatty acid metabolism (Kosa and Ragauskas, 2011; Lennen and Pfleger, 2013; Meng et al., 2009), metabolic engineering (Fischer et al., 2008; Lee et al., 2010; Shi et al., 2011) recovery of biomass and bioproducts (Grima et al., 2003), and scale-up concerns (Brigham et al., 2011; Cuellar et al., 2013).

4. Oleaginous microbes

While biodiesel is most commonly extracted from Triacylglycerols (TAG) stored in plants or animal fats, prokaryotes can also accumulate storage lipids and some species can synthesize TAGs by utilization of organic compounds (Alvarez and Steinbuchel, 2002; Holder et al., 2011). Historically, the appeal of microbial oils was due to a need for an alternative to plant oils and specific polyunsaturated fatty acids for human consumption (Wynn and Ratledge, 2005). The economics of most microbial oil has affected the feasibility of industrial production, but with the advancement of fermentation technology, microbial oil (primarily from fungi and yeast) can be produced in quantities equivalent to acres of agricultural land (Alvarez and Steinbuchel, 2002; Wynn and Ratledge, 2005). Microbial oils offer the unique advantage of being cultivated in a controlled environment, which results in consistency and reproducibility in biodiesel production (Fortman et al., 2008).

4.1. Microbial lipids

Oleaginous microbes are those that accumulate more than 20 percent of their biomass as lipid (Wynn and Ratledge, 2005). These lipids, which can range from 20% to over 70% of the cell's biomass, are usually TAGs and stored intracellularly as a reserve supply of carbon and energy (Alvarez and Steinbuchel, 2002; Wynn and Ratledge, 2005). TAGs are water-insoluble triesters of glycerol with fatty acids that have a higher caloric value than carbohydrates and proteins, thus providing an efficient energy reserve, as TAGs yield much more energy when oxidized (Alvarez and Steinbuchel, 2002). In general, TAG accumulation occurs after synthesis of phospholipids during the exponential growth phase and when cellular growth is impaired during the stationary phase under excess carbon and limited nitrogen conditions (Ratledge and Wynn, 2002). Several studies have shown that lipid accumulation in oleaginous microbes occurs after nitrogen exhaustion in the medium (Alvarez et al., 1996; Alvarez and Steinbuchel, 2002; Gouda et al., 2008; Kurosawa et al., 2010; Packter and Olukoshi, 1995; Silva et al., 2010). Phosphorus limitation has also been shown to influence lipid accumulation in oleaginous yeasts grown in high C:P ratios (Gill et al., 1977) and in high C:P ratios on nitrogen-rich substrate (Wu et al., 2010).

Table 1 shows a list of oleaginous species from various microbial groups that have been recognized to accumulate large amounts of intracellular lipids, which could be potentially used for biodiesel production. The types of lipids produced also vary between prokaryotes and eukaryotes. Eukaryotes like yeasts, fungi, and algae usually produce polyunsaturated fatty acid triacylglycerols similar to vegetable oils that can be used for biodiesel or renewable diesel production (Losel, 1988; Wood, 1988). In oleaginous fungi such as *Mucor circinelloides*, the total lipids are only composed of 14% TAG; however, free fatty acids and phospholipids include 32% and 21% of the total storage lipids, respectively (Vicente et al., 2009). It has been also observed that many algal species accumulate substantial amounts of neutral lipids (20–50%

w/w), majority of which is TAG (Hu et al., 2008). In contrast, most prokaryotic bacteria tend to make particular lipids such as glycolipids, lipoproteins, and wax esters (Anderson and Dawes, 1990; Wilkinson and Ratledge, 1988; O'Leary and Wilkinson, 1988). However, TAG accumulation has been revealed in a few bacterial strains to the *Actinomycetes* group such as species of *Mycobacterium*, *Streptomyces*, *Rhodococcus*, and *Nocardia* (Alvarez and Steinbuchel, 2002; Shields-Menard et al., 2015). TAG biosynthesis has been discovered only in aerobic heterotrophic bacteria and cyanobacteria, precisely the *Actinomycetes* group containing the genera *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia*, or *Gordonia*.

The carbon source for cell growth and TAG accumulation can vary, which results in variability of the fatty acid composition (Alvarez and Steinbuchel, 2002). The oleaginous yeast, *R. glutinis*, was shown to achieve lipid production using glucose and xylose with lignocellulosic inhibitors, glycerol, and waste-water streams (Zhang et al. 2011; AmirSadeghi et al., 2015; Karamerou et al., 2017). Similarly, *R. toruloides* was shown to accumulate over 60% of its cell dry weight as lipid using glucose as the carbon source (Tchakouteu et al., 2017). Several species of the fungus, *Mordellistena*, has shown over 40% lipid accumulation in glycerol and in a mixture of glucose and xylose (Harde et al., 2016; Papanikolaou et al., 2017). *Rhodococcus* species are well-known oleaginous bacteria that can tolerate a wide array of carbon sources and accumulate lipid. Most recently, *Rhodococcus opacus*, *rhodochrous*, and *jostii*, has shown almost 50% lipid accumulation using corn stover waste, model lignocellulosic compounds, and benzoate, respectively (Le et al., 2017; Shields-Menard et al., 2015, 2017; Amara et al., 2016). The diversity of carbon sources that promote lipid accumulation in yeast, fungi and bacteria suggests potential advantageous opportunities for microbial oil production in the biofuel industry.

4.2. Lipid synthesis in oleaginous yeasts and bacteria

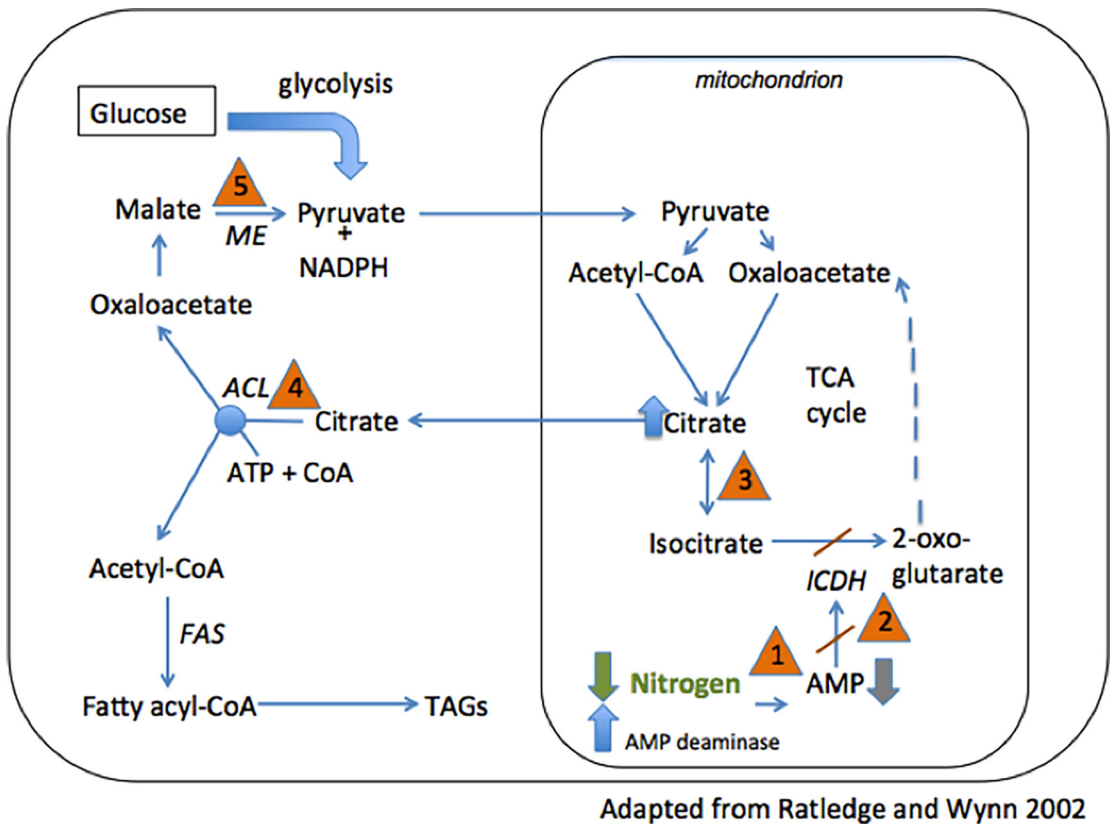
The fundamental requirement for lipid accumulation is a slow growth rate of the cells that will allow excess carbon to be assimilated faster than it can be converted for growth. The slow growth rate implies nutrient limitation and therefore stationary phase has been reached. During stationary phase, cells are still metabolically active and can produce secondary metabolites (those not required for growth).

Mechanisms of lipid accumulation have been well studied in yeasts. The first identifiable metabolic event following nitrogen limitation was observed in yeast in the late 1970s and has been extensively reviewed (Ratledge and Wynn, 2002). As nitrogen is depleted, intracellular adenosine monophosphate (AMP) concentration decreases immediately before lipid accumulation begins. As nitrogen is depleted, AMP deaminase (a nitrogen scavenging enzyme) activity increases, causing a decrease in AMP (Fig. 2, triangle 1). AMP is essential for isocitrate dehydrogenase (ICDH) to catalyze the reaction of isocitrate to 2-oxoglutarate. As AMP concentration decreases, ICDH activity also decreases (Fig. 2, triangle 2). Isocitrate equilibrates with citrate by the enzyme aconitase (Fig. 2, triangle 3). The citrate is transported out of the mitochondria into the cell where it is cleaved by ATP:citrate lyase (ACL) to oxaloacetate and acetyl-CoA (Fig. 2, triangle 4). ACL was the first identified enzyme that influences the different metabolic activity exhibited between oleaginous yeast and nonoleaginous yeast. Acetyl-CoA can then be used for fatty acid synthesis and TAG accumulation, assuming the proper enzymes are available. Wynn and Ratledge have also shown malic enzyme (ME), which converts malate to pyruvate and NADPH, to be of importance in achieving high concentrations of NADPH for fatty acid synthesis and lipid accumulation (Ratledge, 2002; Wynn and Ratledge, 2005).

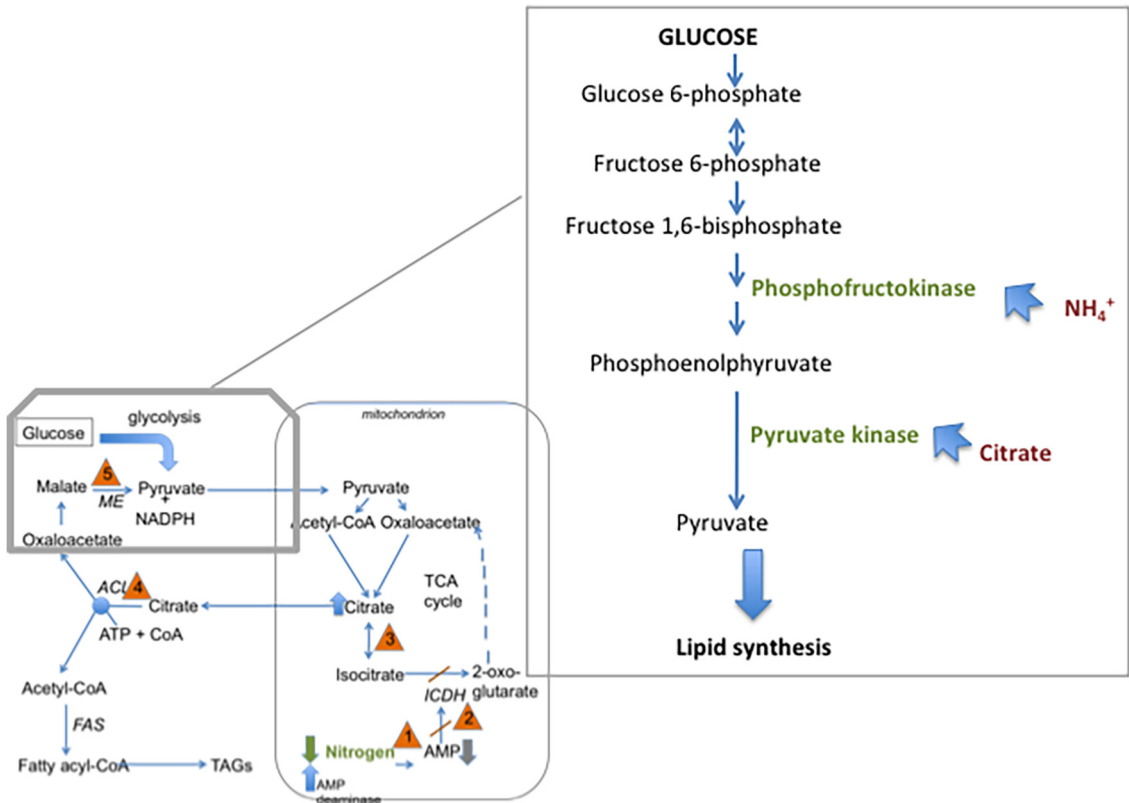
While nitrogen limitation initiates a metabolic shift to favor TAG accumulation in oleaginous microbes, excess glucose (in a high C:N ratio) must still be assimilated (Fig. 3). A key enzyme in glycolysis is phosphofructokinase (PFK), which can be inhibited by high concentrations of citrate. As previously described, nitrogen depletion

Table 1
Lipid content in various oleaginous microorganisms using various waste streams.

Microbial species	Oil Content (% w/w)	References
<i>Algae</i>		
<i>C. vulgaris</i>	56.6	Liu et al. (2008)
<i>C. emersonii</i>	63	Illman et al. (2000)
<i>C. minutissima</i>	57	Illman et al. (2000)
<i>C. sorokiniana</i>	22	Illman et al. (2000)
<i>Yeast</i>		
<i>L. starkeyi</i>	52.6	Kong et al. (2007)
<i>C. curvatus</i>	34.6	Zhang et al. (2011)
<i>Y. lipolytica</i>	58.5	Tsigie et al. (2011)
<i>R. glutinis</i>	53	Karamerou et al. (2017)
<i>R. toruloides</i>	64.5	Tchakouteu et al. (2017)
<i>C. echinula</i>	37.6	Du et al. (2007)
<i>C. albidus</i>	65	Meng et al. (2009)
<i>C. potothecoides</i>	46.13	Han et al. (2006)
<i>R. toruloides</i>	37.6	Zhang et al. (2016)
<i>C. curvatus</i>	37.8	Annamalai et al. (2018)
<i>L. starkeyi</i>	38	Rahman et al. (2017)
<i>C. curvatus</i>	56.4	Zhou et al. (2017)
<i>Fungi</i>		
<i>M. isabellina</i>	50.5	Harde et al. (2016)
<i>M. ramanniana</i>	42	Papanikolaou et al. (2017)
<i>M. vinacea</i>	66	Subramaniam et al. (2010)
<i>Bacteria</i>		
<i>R. opacus</i> PD630	42.1	Le et al. (2017)
<i>R. opacus</i> PD630	87	Alvarez et al. (1996)
<i>R. rhodochrous</i>	50	Shields-Menard et al. (2015)
<i>B. alcalophilus</i>	24	Subramaniam et al. (2010)
<i>R. jostii</i>	55	Amara et al. (2016)
<i>R. opacus</i>	27	Mahan et al. (2017)
<i>R. opacus</i>	29	He (2017)



Adapted from Ratledge and Wynn 2002.



Adapted from Ratledge and Wynn 2002.

ultimately results in an increase of citrate. However, phosphofructokinase can form a stable complex with NH_4 and remain active so that carbon can still be assimilated (Wynn et al., 2001). Furthermore, pyruvate kinase is regulated by citrate to ensure a continuous flow of carbon to pyruvate (Evans and Ratledge, 1984).

Similarly, when nutrient limitation occurs in some bacteria, cell growth is halted and lipid accumulation can occur (Alvarez and Steinbuchel, 2002). TAG accumulation occurs predominantly in stationary phase in many organisms (Alvarez et al., 2000) as fatty acid biosynthesis shifts from phospholipids for cell proliferation to TAG storage (Alvarez and Steinbuchel, 2002). Diacylglycerol acyltransferase (DGAT) has been shown to be a key enzyme in TAG biosynthesis (Alvarez et al., 2000; Davila Costa et al., 2015). Research to identify other key enzymes involved in bacterial lipid biosynthesis is still ongoing (Davila Costa et al., 2015).

5. *Rhodococcus opacus* as a model oleaginous bacterium

Bacteria belonging to the gram-positive Actinobacteria phylum, such as *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia*, or *Gordonia*, can synthesize and accumulate TAGs (Alvarez and Steinbuchel, 2002). Of these bacteria, some *Rhodococcus* species have been characterized for TAG accumulation (Alvarez et al., 2008; Alvarez and Steinbuchel, 2002; Hernandez et al., 2013; Silva et al., 2010) and have been shown to accumulate up to 80% of cellular biomass as lipids (Alvarez et al., 1996).

Rhodococcus opacus PD630, the first *Rhodococcus* species to be labeled oleaginous, can accumulate variable amounts of TAGs in relation to dry cell weight when grown on different substrates: 76% with gluconate; 38% with hexadecane; and 87% with olive oil (Alvarez et al., 1996). Lipids are stored within inclusion bodies that form intracellularly in *R. opacus* PD630. These inclusions are characterized as having a definite shape, suggesting the presence of a surface membrane as well as the associated proteins involved in inclusion formation (Alvarez et al., 1996). TAGs accumulate intracellularly during periods of starvation, ensuring survival in rapidly changing, harsh environments (Alvarez and Steinbuchel, 2002). Furthermore, inexpensive feedstocks like organic wastes or agro-industrial waste from carob, orange, and sugarcane molasses can be used for TAG accumulation in *R. opacus* PD630 (Gouda et al., 2008). High density cell cultivation of *R. opacus* PD630 obtained high TAG concentrations in bioreactors when sugar beet molasses and sucrose were used as substrates (Voss and Steinbuchel, 2001). TAG accumulation from growth on a variety of carbon sources (Alvarez and Steinbuchel, 2002; Holder et al., 2011) make *R. opacus* a potential candidate for biotechnological application (Gouda et al., 2008; Voss and Steinbuchel, 2001), while its catabolic versatility has made *Rhodococcus* species popular in bioremediation (Larkin et al., 2005; Martinkova et al., 2009). The potential to couple the utilization of diverse substrates with TAG accumulation in *R. opacus* offers a promising approach to alternative fuels.

5.1. *Rhodococcus rhodochrous*: Potential as oleaginous bacterium

Rhodococcus rhodochrous was first described in 1891 (Overbeck, 1891) as a generic term for a group of similar strains that were ultimately reclassified as *Mycobacterium rhodochrous* (Gordon, 1966) and then *Rhodococcus rhodochrous* (Goodfellow and Alderson, 1977). *Rhodococcus rhodochrous* is a red pigment-forming *Rhodococcus* species most notably used for the industrial production of acrylamide from acrylonitrile (Kobayashi and Shimizu, 1998; Yamada and Kobayashi, 1996). Because of a diverse metabolism, the degradative capability of *R. rhodochrous* that can utilize polychlorinated biphenyls, hydrocarbons, and other aromatic compounds as a sole carbon source has been extensively reviewed (Martinkova et al., 2009).

R. rhodochrous contains mycolic acids, which has enabled this bacterium to be utilized as a model for studying toxic glycolipids produced

by other *Mycobacterium*, *Nocardia*, and *Rhodococcus* species (de Almeida and Ionedá, 1989; Ionedá and de Almeida, 1991). Neutral plus fatty acids, total glycolipids, and high polar lipids isolated from *R. rhodochrous* after growth on glucose, galactose, or mannose and were shown to be differentially toxic in the mouse model (de Almeida and Ionedá, 1989). *R. rhodochrous* showed significant growth in glucose (with nitrogen) after 120 h and produced 6% diethyl ether-soluble lipids (de Almeida and Ionedá, 1989). Sorkhoh et al. (1990) characterized lipids of *R. rhodochrous* after growth on dodecane and glucose. As *R. rhodochrous* is a hydrocarbon degrader, more total lipids were observed from growth on dodecane than glucose and included the lipid classes of sterols and diacylglycerophosphocholines (Sorkhoh et al., 1990). *R. rhodochrous* has also been shown to produce fatty acids during total mineralization of hexadecane and octadecane (Rodgers et al., 2000). These studies have shown that diversity in hydrocarbons used as carbon sources impact the production and accumulation of lipids in *R. rhodochrous*.

Rhodococcus rhodochrous ATCC 21198, originally named *Nocardia paraffinica*, is patented for its utilization of gaseous hydrocarbons, including propane, ethane, and butane (Tanaka et al., 1973). Previous research has shown that *Rhodococcus* species grown on propane are induced for co-metabolic degradation of vinyl chloride. Babu and Brown (1984) identified a new propane oxygenase involved in propane metabolism by *R. rhodochrous* ATCC 21,198 and more recently, vinyl chloride degradation by a *R. rhodochrous* propane oxygenase was also shown (French, 2000).

R. rhodochrous lipid accumulation for the purpose of FAMES and biodiesel production was characterized using glucose as a carbon source (Shields-Menard et al. 2015). *R. rhodochrous* was shown to be an oleaginous bacterium, accumulating over 50% of cell mass as lipid when grown using glucose as a carbon source. The lipid profile revealed the presence of phospholipids, mono-, di-, and triglycerides, and wax esters and the fatty acid methyl ester profile showed a prevalence of palmitic and oleic methyl esters, consistent with the characteristics of other oleaginous *Rhodococcus* species. The versatile metabolism of *R. rhodochrous* allows for degradation of several environmental contaminants, persistent compounds, and potentially lignin-derived chemicals. *R. rhodochrous* was recently shown to tolerate model lignocellulosic hydrolysate inhibitors such as phenol and other model lignin compounds for lipid accumulation (Shields-Menard et al. 2017). Proteomic analyses identified an abundance of phenol degradation enzymes present in cultures containing phenol and glucose with phenol as well as an abundance of enzymes involved in lipid synthesis and the central metabolism of *R. rhodochrous* (Shields-Menard et al. 2017).

6. Conclusion

The increasing global demand for liquid energy requires investigation and development of alternative fuels while also mitigating rising environmental concerns. The volatile oil market, energy security, and greenhouse gas emissions have led the United States to implement renewable fuel policies with a research focus on novel substrates that can be repurposed as fuel. Oleaginous microbes can accumulate lipids intracellularly when grown on various carbon substrates and are relevant towards the advancement of biodiesel feedstocks as well as the displacement of other petroleum-based products. Lignocellulosic biomass is a renewable resource and can serve as a substrate for microbial lipid production.

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